Stress responses and sugar metabolism in Bacillus subtilis

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Chapter 3

Production and secretion stress caused by overexpression of heterologous α-amylase leads to inhibition of sporulation and a prolonged motile phase in *Bacillus subtilis*

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ABSTRACT

Transcriptome analysis was used to investigate the global stress response of the Gram-positive bacterium *Bacillus subtilis* caused by overproduction of the well-secreted AmyQ α-amylase from *Bacillus amyloliquefaciens*. Analyses of the control and overproducing strains were carried out at the end of exponential growth and in stationary phase, when protein secretion from *B. subtilis* is optimal. Among the genes that showed increased expression were *htrA* and *htrB*, which are part of the CssRS regulon that responds to high-level protein secretion and heat stress. The analysis of the transcriptome profiles of a *cssS* mutant compared to the wild-type, under identical secretion stress conditions, revealed several genes with altered transcription in a CssRS-dependent manner, for example *citM*, *ylxF*, *yloA, ykoJ* and several genes of the *flgB* operon. However, a high affinity CssR-binding was only observed for *htrA* and *htrB*, and possibly for *citM.* In addition, the DNA macroarray approach revealed that several genes of the sporulation pathway are downregulated by AmyQ overexpression, and a group of motility-specific (σ^D-dependent) transcripts were clearly upregulated. Subsequent flow cytometric analyses demonstrate that upon overproduction of AmyQ as well as of a non-secretable variant of the α-amylase, the process of sporulation is severely inhibited. Similar experiments were performed to investigate the expression levels of the *hag* promoter, a well-established reporter for σ^D-dependent gene expression. This approach confirmed the observations based on our DNA macroarray analyses and led us to conclude that expression levels of several genes involved in motility are maintained at high levels under all conditions of α-amylase overproduction.

Introduction

The Gram-positive bacterium *Bacillus subtilis* is capable of secreting high amounts of endogenous proteins into the extracellular medium [268]. Therefore, this bacterium and its relatives are often exploited as hosts for the production and secretion of heterologous industrially interesting enzymes. Secretion of heterologous proteins in large quantities has been shown to lead to the unfavourable condition of protein misfolding and subsequent degradation [216]. In *B. subtilis*, accumulation of misfolded proteins at the membrane-cell wall interface is sensed by the CssRS two-component system, which consists of the membrane-embedded sensor kinase CssS and the response regulator CssR [108]. This system responds to high-level protein secretion and heat stress by phosphorylation of CssR which, in turn, activates transcription of the monocistronic *htrA* and *htrB* genes [48]. The CssRS system has also been shown to regulate the expression of its own operon [48,129]. HtrA and HtrB are membrane-bound serine proteases whose major functions are degradation of misfolded and aggregated proteins. Expression of both proteases is induced upon excessive expression of secretory proteins or a temperature increase [187]. Thus, the CssRS regulon forms a quality control and defence system when cells are confronted with secretion stress. The members of the CssRS quality control system become involved at that stage and take action in two possible manners, either by acting as a chaperone aiding refolding or preventing unfolding, or by HtrA/B-dependent proteolysis.

Recently, proteomic studies have been performed to investigate the global effects of secretion stress [7]. These studies showed that HtrA is also present in the culture supernatant and, in addition to its protease activity, can act as a molecular chaperone. In addition, global effects of secretion stress in *B. subtilis* were also investigated by transcriptome analysis during the exponential growth phase. This study, however, only revealed differential expression of relatively few genes, including the genes encoding the HtrA and HtrB proteases [109].

Because most of protein secretion in *B. subtilis* takes place during the onset of the stationary phase of growth [21,99], we set out to examine the global transcriptional response of *B. subtilis* under secretion stress conditions at this specific growth phase. Secretion stress was applied by overproducing the well-secreted AmyQ α-amylase from *B. amyloliquefaciens*. Besides examining secretion stress in wild-type cells, we compared transcriptome profiles of a *cssS* mutant strain under conditions of high-level AmyQ production. In this work, we have identified and verified putative novel members of the CssRS regulon and we dissected direct and indirect effects of α-amylase overproduction and protein secretion in stationary phase cultures. Our study reveals that upon overproduction of a non-secreted α-amylase, as well as the secreted wild-type variant, the process of sporulation is severely inhibited. In addition, we show that expression levels of genes involved in motility are maintained at high levels under all conditions of α-amylase overproduction.

Materials and methods

Bacterial strains and growth conditions

B. subtilis 168 with an integrated spectinomycin (*sp*) marker in the *pks* locus (*B. subtilis* 168::*sp*) [86] and *B. subtilis* 168 *cssS*::*sp* [108] were used for transcriptional analyses (see Table 1). *B. subtilis* 168::*sp* was used to avoid possible effects on the transcriptional profiling due to the presence of the spectinomycin gene alone, as was shown by Hamoen *et al*., and to be able to compare the obtained data of this strain with that of the *cssS* insertion mutant. Both strains were transformed with either the pUB110 (empty vector) [48] or the pKTH10 plasmid (pUB110 derivative containing the *amyQ* α-amylase gene of *B. amyloliquefaciens)* [192].

Table 1. Bacterial strains and plasmids.

Strains and plasmids	Genotype	Reference
B. subtilis		
168	trpC2	[137]
168::sp	168, *pks*::*sp*, Sp^r	[86]
IIA-gfp	168, P_{spoIIA}-gfp, Cm^r	[256]
hag-gfp	168, P_{hag}-gfp, Cm^r	This work
IIA/E	168, P_{spoIIA}-gfp, Cm^r; pUB110	This work
IIA/Q	168, P_{spoIIA}-gfp, Cm^r; pKTHM10	This work
IIA/QAla	168, P_{spoIIA}-gfp, Cm^r; pKTHM102	This work
hag/E	168, P_{hag}-gfp, Cm^r; pUB110	This work
hag/Q	168, P_{hag}-gfp, Cm^r; pKTHM10	This work
hag/QAla	168, P_{hag}-gfp, Cm^r; pKTHM102	This work
CB100	*sigD*::cat, Cm^r	[164]
Sik243	*spo0A*::Em, Em^r	[111]
BV2001	*cssS*::*sp*, Sp^r	[108]
Plasmids		
pUB110	Km^r	
pKTH10	Km^r , pUB110 derivative containing the α-amylase gene (*amyQ*) of *B. amyloliquefaciens*	[192]
pKTHM10	Km^r , pKTH10 derivative	[283]
pKTHM102	Km^r , pKTHM10 with the Ala-rich signal peptide of AmyQ	[283]

B. subtilis strains were grown in TY medium (1% tryptone, 0.5% yeast extract and 1% NaCl) at 37°C, 250 rpm containing the appropriate antibiotics. Antibiotic concentrations were: kanamycin (10 μg/ml), spectinomycin (100 μg/ml) and chloramphenicol (5 μg/ml).

Strain construction

To construct plasmid pGFP-hag, a PCR with primer pair hag-F and hag-R (see Table 2) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment containing the promoter region of the *hag* gene was subsequently cleaved with *Hin*dIII and

*Eco*RI, and ligated into the corresponding sites of pSG1151, in that way generating a fusion with the *gfpmut1* gene [144]. *B. subtilis* strain hag-gfp was obtained by Campbell-type integration of plasmid pGFP-hag into the chromosome of *B. subtilis* 168. Transformants were selected on TY agar plates containing chloramphenicol (5 μg/ml), after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown).

Table 2. Primer sequences.

Primer	Sequence (5' > 3')	Target fragment	Restriction site
hag-F	GGGATCAAGTGAAGCTTGGAATTGACG	*hag*	*Hin*dIII
hag-R	CGGAATTCCATTTCCTCTCCTCCTTGAATATGTTGTTAAGGCACGTCC	*hag*	*Eco*RI
QE60-cssR-F	CATGCCATGGCATACACCATTTATCTAGTTGAAGA	*cssR*	*Nco*I
QE60-cssR-R	CGGGATCCTGATGACATCATCCTGTAGCCGAAACCGTA	*cssR*	*Bam*HI
QE30-cssR-F	CGGGATCCTTGTCATACACCATTTATCTAGTTGAAG	*cssR*	*Bam*HI
QE30-cssR-R	CCGAGCTCTTATCATGATGACATCATCCTGTAGCCGAAA	*cssR*	*Sac*I
htrA-EMSA-F	AACGGATCAGCCGATACGTT	P*htrA*	None
htrA-EMSA-R	TCATCACGATAGTTATCCAT	P*htrA*	None
htrB-EMSA-F	CGTCAGCAGTTCATTCAG	P*htrB*	None
htrB-EMSA-R	CCATCACGTCGATAATCC	P*htrB*	None
citM-EMSA-F	AGGTCACCTCCTCACCTGAA	P*citM*	None
citM-EMSA-R	CATGAGAAAGCCTAAGATTGCTAAC	P*citM*	None
flgB-EMSA-F	TGTATCGTTCCAGAAAATAAGC	P*flgB*	None
flgB-EMSA-R	TCAGGGCGAGAAATGTAGTTC	P*flgB*	None
ykoJ-EMSA-F	TGCCGATCAAATCAGCAG	P*ykoJ*	None
ykoJ-EMSA-R	TTGTGAGCCCCTCCTTTGT	P*ykoJ*	None
yloA-EMSA-F	CCAGAAGCACAAGCACCATA	P*yloA*	None
yloA-EMSA-R	GTATGTAAACATGCCATCAAACG	P*yloA*	None
pK-F	AATCTATCGACATATCCTGCAA	P*comK*	None
K-FP-R	GGAATTCTTGCGCCGTTCACTTCATAC	P*comK*	None

RNA isolation, preparation of labelled cDNA and hybridization

Cells were grown overnight in 10 ml of TY medium with kanamycin (10 μg/ml) and then diluted to an optical density at 600 nm of 0.1 in 40 ml of TY medium containing kanamycin. Samples for transcriptome analyses were collected at the late exponential growth stage (one hour before the transition point) and 3 hours upon entry in the stationary growth phase. Three independent cultures of each strain were used and cells were sampled for macroarray experiments. RNA was isolated by spinning down cells from 4 ml of culture, subsequent cell disruption, phenol-chloroform extraction, followed by RNA purification with a High Pure RNA isolation kit from Roche, as described previously [86]. RNA was eluted with 50 μl of elution buffer and subsequently quantified with GeneQuant (Amersham) and the RNA integrity was checked on agarose gels. 4 μg of RNA as a template and 1 pmol of ORF specific primers (Eurogentec) were used for the reverse transcriptase reaction with SuperscriptII (Gibco BRL). The detailed protocols of reverse transcription and purification of ^{33}P-labelled cDNA are outlined by Hamoen *et al*. [86]. Labeled cDNA was hybridized to *B. subtilis* Panorama™ Arrays (Sigma-Genosis), according to the manufacturer's instructions. The

membranes were exposed to Cyclone phosphoimager screens (Packard) for approximately 2.5 days and signals were quantified with Array-Pro Analyzer 4.5 (Media Cybernetics).

Data analysis

Duplicate spots were averaged in Array-Pro software (Media Cybernetics, Inc.) and the signal was normalized after background subtraction by calculation of the percentage of total signal per gene using Microsoft Excel. Outstandingly high signals (for example whole genomic DNA control spots which act as a positive control and always show very strong signals) were excluded from calculations of the total signal. Statistical significance of the obtained gene expression ratios was assessed by the Cyber-T program [149]. Genes with a Cyber-T *p*-value lower than 0.05 are discussed in this paper. The array data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE8014.

SDS-polyacrylamide gel electrophoresis and Western blotting

To detect the precursor and mature forms of AmyQ in cells and growth medium, 1 ml of culture was centrifuged and the cell pellet was washed once with fresh medium. After separation by SDS-PAGE, proteins were transferred to Immobilon-PVDF membranes. The visualization of AmyQ protein was carried out with specific anti-AmyQ antibodies and horseradish peroxidase-anti-rabbit IgG conjugates and ECL immunobloting detection reagents (Amersham).

Amylase activity assay

Samples from culture supernatants were diluted 100 times and were subjected to amylase activity quantification with the EnzCheck Ultra Amylase Assay Kit (Molecular Probes), according to the manufacturer's instructions. Assays were carried out in 96-well microtiter plates in 100 µl total volume using 50 µl diluted supernatants. Reactions contained 200 µg/ml DQ starch substrate and were carried out in 100 mM MOPS at pH 6.9. The degradation of the substrate by amylase yields highly fluorescent fragments and fluorescence was monitored at room temperature every 5 min for 1h with a microplate reader TECAN (GENios) using standard fluorescein filters. Samples were taken from three independent experimental replicates.

Flow cytometry

Cells were 100 times diluted in 0.2 µM of filtered minimal medium [6] and directly measured on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, NL), operating an argon laser (488 nm) as essentially described by Veening *et al* 2005 [256]. For each sample, at least 20,000 cells were analyzed. Data containing the green fluorescent signals were collected by a FITC filter and the photomultiplier voltage was set between 700 and 800 V. Data was

captured using System II software (Beckman Coulter) and further analyzed using WinMDI 2.8 software (http://facs.scripps.edu/software.html). Figures were prepared for publication using WinMDI 2.8 and Corel Graphics Suite 11. To distinguish background fluorescence from GFP-specific fluorescence, the parental strain *B. subtilis* 168 was also analyzed with each flow cytometric experiment.

Construction and purification of 6xHis-tagged CssR

Plasmids encoding CssR protein with a 6xHis-tag were constructed as follows. For C-terminally tagged protein, the *cssR* open reading frame was amplified by PCR using primers QE60-cssR-F and QE60-cssR-R (see Table 2) and Phusion polymerase (Finnzymes). Chromosomal DNA of *B. subtilis* strain 168 was used as a template. The product of this reaction was digested with *Nco*I and *Bam*HI, and cloned into similarly digested pQE60 plasmid (Qiagen), yielding plasmid pQE60-cssR.

To obtain an N-terminally tagged CssR protein, the *cssR* open reading frame was amplified by PCR using primers QE30-cssR-F and QE30-cssR-R. The product of this reaction was digested with *Bam*HI and *Sac*I, and cloned into similarly digested pQE60 plasmid (Qiagen), yielding plasmid pQE30-cssR.

To induce expression, *E.coli* JM109 carrying pQE60-cssR or pQE30-cssR was diluted 1:100 to fresh medium from an overnight culture. At an OD600 of 0.6, expression was induced by the addition of 1mM of IPTG. Cells were harvested after an additional two hours of growth, after which protein was purified as described elsewhere (Smits *et al*, in press). The purity of the protein was estimated to be >95% pure on the basis of Coomassie stained SDS-PAGE gels. After purification, the protein was dialysed against dialysis buffer (20 mM Tris-HCl pH8, 1mM EDTA, 10 mM $MgCl_2$, 0.2 M NaCl, 1 mM beta-mercaptoethanol, 5 mM imidazole, 7% glycerol). The concentration of protein was determined on the basis of A280 using the Nanodrop machine, with 0.1% (1g/L) giving a value of 1.272 (ExPASy Protparam Tool; http://www.expasy.org/tools/protparam.html).

Electrophoretic mobility shift assays

To establish direct binding of CssR to putative target promoters, gel shift experiments were carried out as described before [4]. In our experiments, we observed no significant difference in affinity between C- and N-terminally His-tagged proteins. Subsequent experiments were therefore carried out using only N-terminally His-tagged protein. The fragments obtained corresponded to basepairs -339 to +19 (*htrA*), -335 to +19 (*htrB*), -333 to +26 (*citM*), -191 to +25 (*flgB*), -320 to +0 (*ykoJ*) and -312 to +32 (*yloA*) compared to the start of the open reading frame as annotated in SubtiList R16.1 (http://genolist.pasteur.fr/SubtiList/). As a negative control the promoter of *comK* (-201 to +47) was amplified. All primer sequences are available in Table 2.

Results

Transcriptomics of secretion stress in late exponential and stationary phase cells

AmyQ encodes the α-amylase from *Bacillus amyloliquefaciens* that contains a Sec-type signal sequence. This protein has been shown to trigger a specific secretion stress response when overexpressed in *B. subtilis* [108]. DNA macroarray analysis was used to compare transcriptional profiles of *B. subtilis* 168::*sp* containing plasmid pUB110 (empty vector) with those of *B. subtilis* 168::*sp* containing plasmid pKTH10 (AmyQ overexpression) grown in TY broth. Samples were taken for transcriptome analyses during late exponential (one hour before the transition point) and stationary growth (3 hours after the transition point) phases of growth. Amylase-dependent starch degradation on TY-agar plates and Western blot analysis of the growth media from the cells used for the macroarray experiment, verified the expected AmyQ overproduction for the pKTH10-containing cells (data not shown).

Differentially expressed genes in the late exponential phase are listed in Table 3. As expected, the highest upregulated genes are *htrA* and *htrB*, encoding the Htr-like proteases. Also moderately elevated transcription levels of the *cssRS* operon, encoding the secretion stress response regulator (CssR) and histidine kinase (CssS), were observed upon AmyQ overproduction, indicating that cells were clearly subjected to secretion stress. In addition, a stimulatory effect was observed for the transcripts of the ribosomal protein RpsB and the genes for the general stress proteins GroEL and DnaK, suggesting that next to secretion stress, also a cytosolic stress, most likely resulting from the presence of misfolded and aggregated proteins, is induced. Moreover, two members of the peroxide stimulon, *ahpF* and *mrgA*, next to the genes involved in cell wall homeostasis (*dltA*, *acpA* and *accC*), showed elevated expression levels. Other genes with known function that were induced upon AmyQ overproduction include *pycA* and *citB* of the TCA cycle and *sodA* and *trxA*, which play an important role in maintaining the redox balance of the cell. The secretion stress directly or indirectly caused increased mRNA levels of four genes with an unknown or predicted function, including *yvfW* encoding a putative iron-sulfur-binding protein and *yvqH* which has similarity to the *E. coli* phage shock protein A that was shown to be induced under anaerobic growth [273] and ethanol stress conditions [193], respectively. Also *ykoJ* and *ydbK* showed increased transcript levels. The former code for a conserved protein of unknown function and the latter is a putative ABC transport system permease protein. Besides the upregulation of a number of genes, some of which have been mentioned above, more than 40 genes were significantly downregulated upon AmyQ overproduction. These genes classify within different functional categories including, amongst others, metabolism of lipids, transport/binding proteins and sporulation (Table 3).

Under natural conditions, most of the secretion takes place during stationary growth phase. Therefore, transcriptome analyses were also performed on AmyQ overproducing and non-overproducing cells in this phase. Again, a strong effect on the transcriptional levels of *htrA*,

htrB, *cssS* and *cssR* was observed (Table 4). Thus, secretion stress also occurs during later stages of growth. From the transcriptional profiling in this growth phase, two main novel findings can be deduced: several genes involved in motility and chemotaxis, including *sigD*, had increased mRNA levels, in contrast to a group of sporulation-related genes, which appeared to have decreased transcription levels (Fig. 1). The list of downregulated sporulation related genes is more extensive, although for some of them the obtained expression ratios were slightly below the statistical cut-offs.

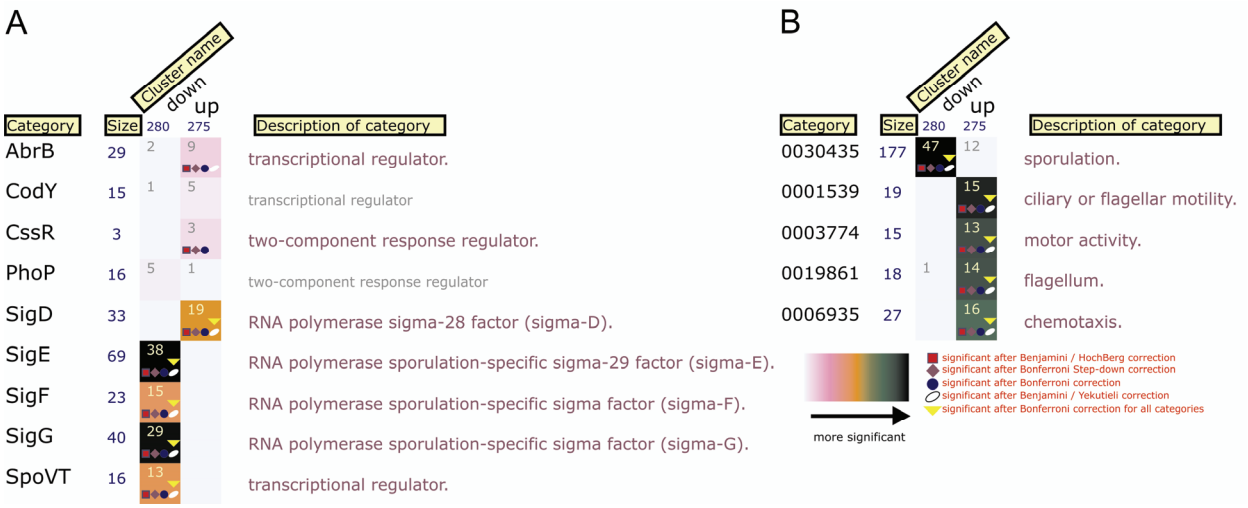

Fig. 1. FIVA analysis of interaction and GO (Gene Ontologies) categories of the transcriptome data.
Genes from DNA-macroarray dataset were divided into up- and down regulated clusters (comparison of gene expression ratios between *B. subtilis* 168 (pKTH10)/*B. subtilis* 168). Panel (A) and panel (B) show "interaction" and GO (Gene Ontology) categories, respectively. The size of each cluster is presented in blue underneath the cluster name. Numbers in each rectangle represent how many genes were up- or downregulated per cluster in each category. The colours of square boxes depict the significance of gene enrichment per category as seen in the legend. Detailed information on significance analyses is available in Blom *et al* [20].

The protein phosphatase encoded by *prpC* (*yloO*) was activated and the product of this gene, together with the PrkC protein kinase, have been shown to be implicated in the sporulation process [158]. Two sporulation genes, *spo0JA* and *spo0E*, had increased transcription levels under the secretion stress but, interestingly, both of them affect sporulation in a negative manner [195]. Expression of several other genes seems also to be affected by the AmyQ overproduction. The products of these genes are associated with different cellular processes such as metabolism of amino acids, lipids and carbohydrates or transport and binding activity (Table 4). For the genes with unknown function, *yloA* and *ykoJ* showed the strongest upregulation (11.2 x and 5.3 x, respectively). Both genes are well conserved among bacterial species. YloA bears similarity to a fibronectin-binding protein as well as to RNA-binding proteins that show homology to the eukaryotic snRNP's. YkoJ was also induced at the earlier time point. This protein contains a putative signal peptide and two PepSY domains which were suggested to have a peptidase regulatory activity in the cell wall proximity [275].

Verification of putative members of the CssRS regulon

As secretion stress clearly affected the expression of the known members of the CssRS regulon, we attempted to identify novel members of this regulon with a focus on the upregulated genes resulting from AmyQ overexpresison. For this purpose we compared the transcriptome profiles of *B. subtilis* 168::*sp* (wild-type) and 168 *cssS*::*sp* (Δ*cssS*), both containing the pKTH10 plasmid. Previous research showed that *htrA* and *htrB* are regulated by the CssRS system and that disruption of *cssS* reduces *htrA* and *htrB* transcription [48,108]. Our transcriptome data confirmed these observations since both genes showed decreased mRNA levels in the *cssS* mutant strain. Beside *htrA* and *htrB,* similar expression patterns from our macroarray approach were observed for *citM* (secondary Mg-citrate transporter), *flgB* operon (flagellar synthesis and chemotaxis), *ylxF*, *yloA* and *ykoJ*.

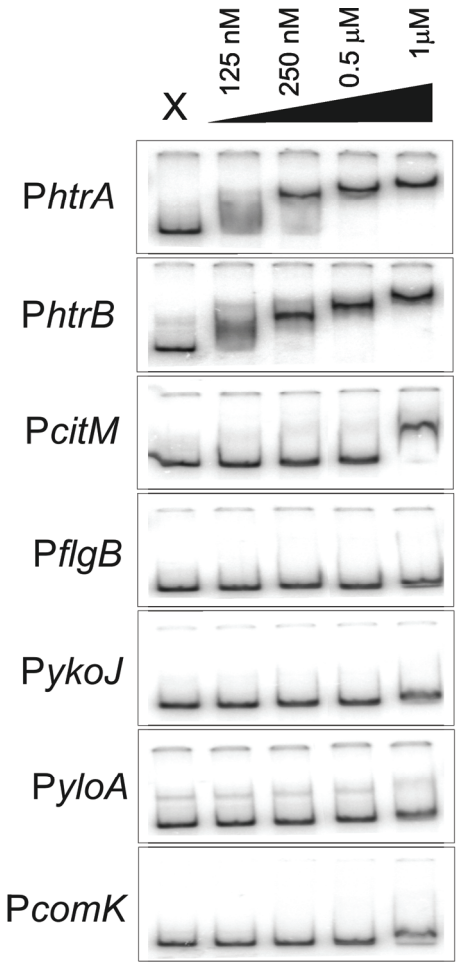

Fig. 2. Binding of CssR-His6 to the indicated promoter regions.
Gel retardation reactions were performed with radiolabeled DNA fragments prepared by PCR and end labeled with ^{32}P. Promoter regions were incubated with increasing concentrations of purified CssR-His6 (see Materials and methods) ranging from 0.125 µM to 1 µM of the protein. In each panel the lane marked with x corresponds to the reaction with no protein added.

Our analysis revealed several genes with altered transcription levels in response to overproduction of AmyQ, in a CssR-dependent manner. Though CssR is a two component regulator, it is unknown if the observed effects are due to direct binding of CssR to the target promoters. In fact, binding of CssR to target promoters has not been reported so far. Therefore, several candidate genes were selected and electrophoretic mobility shift assays

(EMSAs) were carried out, using purified N-terminally His-tagged CssR protein. Phosphorylation of non-tagged CssR by acetyl-phosphate enhances the affinity of the protein for DNA 2-4 fold dependent on the promoter fragment used (E. Darmon, unpublished observations). Figure 2 shows that unphosphorylated, 6xhistidine tagged CssR has a high affinity for the DNA fragments containing the promoters of the *htrA* and *htrB* genes, with an apparent K_D of ~125 nM, therefore, the tagged protein likely mimics the phosphorylated form of the protein. These observations are in good agreement with previous reporter studies that demonstrate the CssR-dependent induction of these genes upon protein overproduction [48,108]. In contrast, unphosphorylated CssR does not to appear to bind strongly to any of the other fragments, with the possible exception of *citM*, for which consistently a reduced mobility was observed at 1μM of CssR protein. We conclude that for the fragments used, only *htrA* and *htrB* contain a high affinity CssR-binding site. As for the other genes, the observed transcriptional changes most likely result from secondary effects.

Sporulation and motility are affected by AmyQ overproduction and secretion

Several genes that were significantly downregulated by AmyQ overexpression belong to the sporulation pathway. This suggests that secretion stress results in inhibition of spore formation. Interestingly, overexpression of a cytoplasmic protein did not affect early sporulation gene expression [118], indicating that secretion stress rather than overproduction stress might be the cause for the observed downregulation. To examine whether the observed downregulation of the sporulation pathway in our transcriptome analysis by AmyQ overproduction was specifically caused by secretion stress, or was a result of an indirect effect caused by AmyQ overproduction, we made use of a sporulation specific reporter strain carrying the *spoIIA* promoter in front of the gene encoding the Green Fluorescent Protein (GFP). The *spoIIA* promoter is directly activated by the key sporulation regulator, Spo0A, and was shown to be a good reporter for cells that initiate sporulation [256]. Plasmids pKTHM10 (AmyQ overexpression), pKTHM102 (AmyQ-Ala overproduction) and pUB110 (empty vector) were introduced in the indicator strain and cells were analyzed for their expression levels by flow cytometry. The pKTHM102 vector is a derivative of pKTHM10 (which is similar to pKTH10) and encodes the AmyQ protein with an Ala-rich signal sequence that renders it inactive in translocation across the cytoplasmic membrane [283]. Importantly, this AmyQ variant does not evoke a secretion stress response [267]. Strains were grown in TY medium and samples were taken at hourly intervals and examined by flow cytometry. As depicted in Fig. 3, strain IIA/Q (P_{spoIIA}-*gfp*, pKTHM10) harbouring an AmyQ overexpression vector with the wild-type signal peptide, was slightly delayed in the activation of the *spoIIA* operon (Fig. 3). A more pronounced effect was observed in cells containing the pKTHM102 plasmid (strain IIA/QAla). Overall, these results show that both the secretion stress and AmyQ overproduction (IIA/QAla) in particular, inhibit the process of sporulation. In addition, this inhibition already occurs at the earliest

stages in spore formation, at the level of phosphorylation of Spo0A, as shown by the single cell analyses.

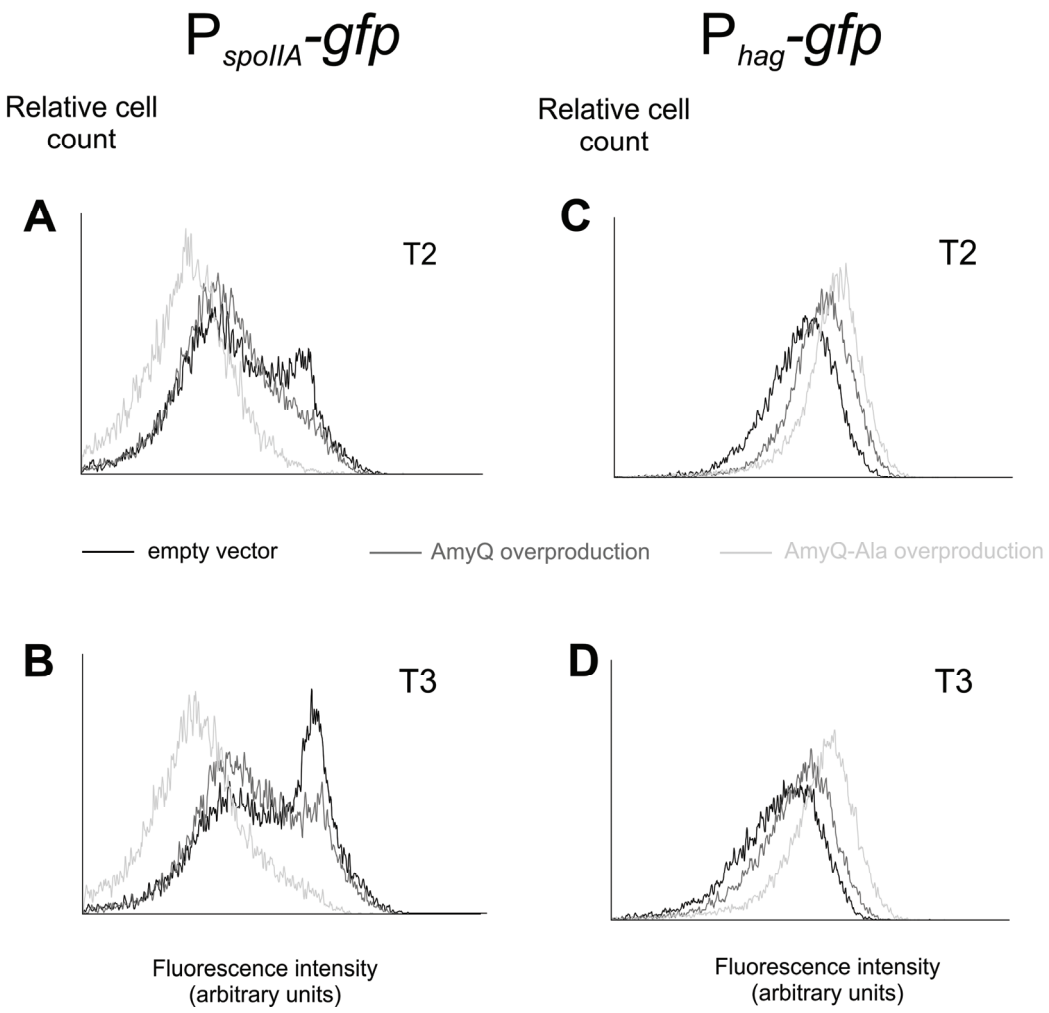

Fig 3. Single cell analysis of sporulation and motility specific gene expression.
Strains were grown in TY medium at 37°C with shaking. Samples for flow cytometric analysis were taken every hour during growth. Two-time points are represented, T2 and T3, which correspond to two and three hours after the entry into stationary phase, respectively. The numbers of cells measured are indicated on the *y* axis and their relative fluorescence is indicated on the *x* axis. (**A-B**) Strains P_{spoIIA}-gfp: IIA/E (empty vector, black line), IIA/Q (AmyQ overproduction, dark grey line) and IIA/QAla (AmyQ-Ala overproduction, light grey line). (**C-D**) Strains P_{hag}-gfp: hag/E (empty vector, black line), hag/Q (AmyQ overproduction, dark grey line) and hag/QAla (AmyQ-Ala overproduction, light grey line).

Another adaptive response that *B. subtilis* utilizes is motility. In this case, cells physically escape from adverse circumstances towards more promising ones to increase their survival chances. Regulation of synthesis of flagella and motility gene expression is known to be orchestrated by an alternative sigma factor, σ^D [96,164]. As described above, we observed a strong upregulation of several genes involved in motility upon AmyQ overproduction. To investigate the increase of flagellar gene expression in *B. subtilis* as a result of protein overproduction, we constructed a strain in which the σ^D-dependent *hag* promoter was fused

to the *gfp* gene (hag-gfp). The *hag* gene codes for the flagellin protein and it was shown before that this gene can be used as a good reporter for studying environmental effects on σ^D-dependent gene expression [169]. The AmyQ overproduction plasmids were introduced in this reporter strain, resulting in strains hag/Q (P_{hag}-*gfp*, pKTHM10), hag/QAla ((P_{hag}-*gfp*, pKTHM102) and hag/E (P_{hag}-*gfp*, pUB110). Cells were grown in TY medium and samples were collected at hourly intervals for flow cytometric analyses. As shown in Fig. 3, the promoter activity of the flagellin gene is the highest for the strain overproducing AmyQ with the Ala-rich signal peptide. This again suggests that the enhanced expression of motility genes is not a direct effect of secretion stress itself, but rather results from AmyQ overproduction.

Effects of *sigD* and *spo0A* deletions on AmyQ overproduction and secretion

The results of the transcriptome analyses have shown that two cellular adaptive processes were clearly affected under the secretion stress conditions. This raised the question whether disruption of these processes would affect heterologous protein secretion by *B. subtilis*.

It has been previously investigated how the yields of protein production can be improved by modification of the components engaged in the late stages of protein secretion, especially the ones influencing the cell wall-associated protease activity or the cell wall composition [268]. To further study whether sporulation and/or motility affect protein secretion in *B. subtilis*, *sigD* (σ^D) and *spo0A* mutations were introduced in a strain containing the pKTHM10 plasmid (AmyQ overproduction).

Mutants of *spo0A* are defective in sporulation since this transcriptional regulator plays a central role in the initiation of this developmental process [195]. The null mutant of σ^D is non-motile and shows reduced levels of autolysins [164]. The production levels of secreted AmyQ in both mutant strains and the parental strain were determined at different time points of growth by means of the EnzCheck Ultra Amylase Assay Kit as described in the Materials and Methods section. In all cases, the amount of active amylase in the culture medium increased from exponential phase (T1) and reached a maximum in the late stationary phase (T4) (Fig 4). However, a clear delay was observed in the *spo0A* mutant, as this strain secreted significantly less amylase than the wild-type strain during exponential phase. In the late stationary phase the difference became minimal between the two strains. More interestingly, under our experimental setup, the *sigD* mutant reached slightly higher levels of the active enzyme at earlier growth phases when compared to the wild-type strain. Again, after four hours upon entry in stationary phase the differences were negligible. These results indicate that when the cells are devoid of the possibility to initiate sporulation or to enter the motile phase, the efficiency and timing of protein secretion by *B. subtilis* are modulated.

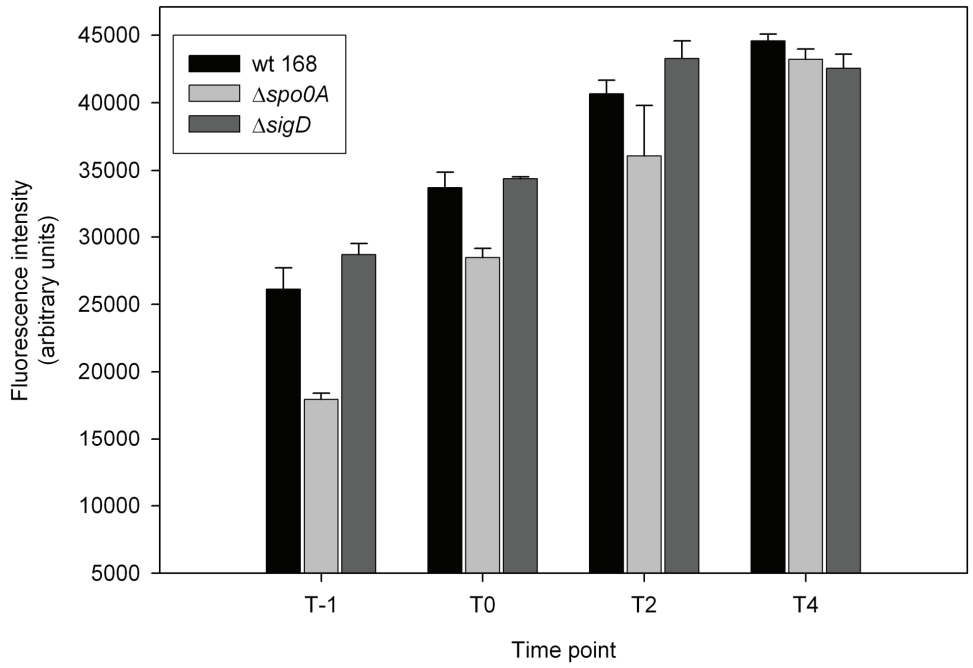

Fig. 4. Activity of secreted amylase from *B. subtilis* 168 wild-type, sporulation- and motility-deficient strains. Amylase activity was quantified in the culture medium of the wild-type strain (black bars), the *spo0A* mutant (light grey bars) and the *sigD* mutant (dark grey bars). All strains were transformed with the pKTHM10 plasmid (AmyQ overproduction) and levels of activity of secreted AmyQ were determined at different time points of growth by using the EnzCheck Ultra Amylase Assay Kit as described in the Materials and Methods section. T0 corresponds to the transition phase of growth. Error bars represent standard errors over three independent biological replicates.

Discussion

Protein secretion, like competence development and sporulation, is one of the post-exponential processes that *B. subtilis* employs as a response to altering growth conditions. The secretion activity is rather low during exponential growth and increases substantially at the onset of stationary phase [200]. It has been shown before that several components of the Sec machinery in *B. subtilis* reach the maximum expression at the end of exponential growth [99], or in the early post-exponential phase [21]. Based on these facts, we set out to study the global cellular response of *B. subtilis* to α-amylase (AmyQ) overproduction and secretion stress during late exponential and stationary phase. In all our macroarray analyses, we observed upregulation of the known targets of the secretion stress regulon, *htrA* and *htrB* (Fig. 1, Table 3 and 4). This validates the quality of the obtained transcriptome data and agrees well with previously published results [48,108]. The comparison of the transcriptional patterns of the control strain and the AmyQ-overproducing strain at the late exponential phase of growth disclosed a rather modest response of *B. subtilis* to the applied stress. This result is in a good accordance with the studies of Hyyrylaïnen *et al*, where the induction of only seven genes upon high-level AmyQ secretion was found [109]. However, our transcriptome analysis at the late exponential phase revealed the activation of a higher

number of genes, which most likely results from the differences in experimental approaches, especially the later sampling in growth in our case. Next to the stimulation of components of the CssRS quality control system, several other genes were weakly induced. Most of these gene products are involved in the stress response evoked by protein misfolding/aggregation events in the cytoplasm, detoxification and fatty acid metabolism, indicating that cells sensed and tried to counteract the adverse conditions.

Our data revealed the CssRS-dependent regulation of several genes, in response to AmyQ overproduction. However, for only two of the (putative) targets (*htrA* and *htrB*), binding of the purified CssR protein to the DNA could be demonstrated, indicating that CssR directly binds to and regulates expression of these operons. How can the apparent CssR-dependent expression of these genes then be explained? Firstly, it is possible that some of the promoter regions tested by EMSA require additional co-factors *in vivo* for a proper binding of CssR. Secondly, phosphorylated CssR might bind different DNA sequences and with other affinities than unphosphorylated CssR. Since our EMSA experiments were carried out with unphosphorylated protein, we cannot exclude that *in vivo* some of the target genes are more strictly dependent on the phosphorylated form of CssR. Another explanation is that the observed effects are indirect and independent of CssR, for instance via increased levels of HtrA, the activity of which was postulated to influence the regulatory effects of a two-component system in *Streptococcus pneumoniae* [222]. Finally, data obtained from a P*ykoJ-gfp* fusion at its native locus, indicates that up-regulation of this gene in response to protein overproduction is indeed CssR-dependent but that promoter activity could not be demonstrated in an area of 500bp upstream of the *ykoJ* open reading frame (reference [186] and unpublished observations). The promoters of *htrA* and *htrB* are remarkably similar, making the *in silico* identification of a CssR binding motif very difficult. However, a Gibbs sampling method [243] identifies a CATTTTTATC motif, that is present in the 300-bp region upstream of both genes. In addition, close matches to this motif (GATTTTTTTC and CATTTTTTTC) can be identified 300-bp upstream of *citM*. The importance of this putative binding sequence remains to be established in future investigations.

The most striking outcome of the transcriptome analysis of stationary phase cells was the influence of protein overproduction on the processes of sporulation and motility. The effect on sporulation was much more pronounced at the later stages of growth and reduced expression levels of many SigE, SigF and SigG regulon members were observed in AmyQ overproducing cells (Fig. 1). Interestingly, the level of *spo0E* transcript (phosphatase of Spo0A~P) in the AmyQ-overexpressing cells is higher than in the wild-type (pUB110) due to which the Spo0A~P pool might be drained. The inhibition of sporulation was confirmed by *gfp*-reporter experiments, and these analyses showed that sporulation is already blocked at the earliest stages (at the level of Spo0A~P) of amylase overproduction (Fig. 3). Using a non-translocated AmyQ variant, the differences between secretion stress and AmyQ production stress were examined. This approach led us to conclude that the impaired sporulation phenotype is most likely not an exclusive and direct effect of secretion stress, but is rather

caused by high-level cellular protein production and accumulation, possibly at the membrane. In fact, overproduction stress seems to inhibit sporulation more efficiently than secretion specific stress (Fig. 3). It is plausible that the unprocessed AmyQ-Ala variant is still targeted to the Sec translocon and thereby leads to a kind of membrane-congestion stress. This type of stress would account for the observed effect on sporulation and motility and would be less severe in case of the wild-type AmyQ variant which is translocated across the cytoplasmic membrane.

Sporulation and protein secretion are multistep- and energy consuming processes and since protein production and secretion take place earlier in the life cycle of *B. subtilis* than endospore formation, it most likely inhibits the latter due to energy constraints. Our data suggests that *B. subtilis* cells sense a variety of environmental and intracellular signals under protein overproduction stress which leads to the decision that commencing the energy-consuming spore development is not appropriate since it is highly unlikely that this process can be completed successfully.

Another survival strategy which *B. subtilis* employs as an adaptive response under unfavourable environmental conditions is motility. Remarkably, our transcriptome profiling revealed that a substantial part of the motility regulon was induced by high-level protein production and secretion. The alternative sigma factor, σ^D, orchestrates the transcription of many genes whose products are in flagellar assembly, motility, chemotaxis and autolysis [96,164]. In a complex medium the amount of the σ^D protein increases during growth, reaching the maximum level at the transition point [169,170]. By single-cell flow cytometric analysis, we show that AmyQ overproduction prolongs the motile phase of *B. subtilis* (Fig. 3). One of the explanations for this prolonged motile phase includes the putative replacement and competition of alternative sigma factors for core RNA polymerase (RNAP) during stationary phase, a phenomenon well-documented for both *E. coli* [66,163] and *B. subtilis* [100,117,151]. The consecutive sporulation-specific sigma factors conduct developmental events during endospore formation [59]. Since sporulation is blocked upon protein overproduction (Fig. 3), SigD does not have to compete with sporulation-specific sigma factors for free RNAP. However, it has been demonstrated that RNAP is present in excess in *B. subtilis* cells and the engagement of anti-sigma factors or other mechanisms, which would give rise to deactivation of σ^A, could be conceived [74]. An extended expression of the motility regulon can provide cells with a rescue machinery under the deficiency of sporulation but, on the other hand, the members of yet uncharacterized σ^D-regulated genes could be also involved in helping cells to survive stress circumstances.

Our transcriptome results, combined with the single cell analyses, predicted that both initiation of sporulation and motility play an important role in combating protein secretion stress. Indeed, when either *spo0A* or *sigD* were mutated, protein secretion was significantly affected (Fig. 4). It is demonstrated that the presence of a functional *spo0A* is required for efficient protein secretion, while the removal of *sigD* moderately but consistently increases secretion (Fig. 4). It has been shown before that a *sigD* mutation causes an increased

extracellular accumulation of an artificial cell wall-binding lipase by an unknown mechanism [128]. Likewise in our experiments, the secretion of enzymatically active AmyQ turned out to be enhanced in the *sigD* mutant, especially in earlier phases of growth, which renders this mutant strain a good host for efficient extracellular protein production (Fig. 4).

Supplementary material

The slide images and raw data mentioned in this manuscript are available from http://molgen.biol.rug.nl/publication/secstress_data/

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Table 3. Differentially expressed genes in response to AmyQ overproduction in the late exponential phase of growth.
[1)]Fold represents relative change in expression levels between the stressed and non-stressed cells.

gene	Fold[1]	Function	Functional group
htrA	22.7	serine protease (heat-shock protein)	Adaptation to atypical conditions
htrB	7.8	serine protease (heat-shock protein)	Adaptation to atypical conditions
rpsB	5.0	30S ribosomal protein S2	Protein synthesis; ribosomal proteins
ahpF	2.6	alkyl hydroperoxide reductase (large subunit) and NADH dehydrogenase	Detoxification
citB	2.5	aconitate hydratase (aconitase)	Metabolism of carbohydrates and related molecules; TCA cycle
groEL	2.4	class I heat-shock protein (chaperonin)	Protein folding
acpA	2.3	acyl carrier protein	Metabolism of lipids
ftsZ	2.2	cell-division initiation protein	Cell division
sodA	2.1	superoxide dismutase	Detoxification
cssR	2.0	two-component response regulator involved in the control of cellular responses to protein secretion stress	Sensors (signal transduction)
cssS	1.9	two-component sensor histidine kinase involved in the control of cellular responses to protein secretion stress	Sensors (signal transduction)
accC	1.9	acetyl-CoA carboxylase subunit (biotincarboxylase subunit)	Metabolism of lipids
yerP/swrc	1.9	involved in the efflux of surfactin	Detoxification
mrgA	1.8	DNA-binding stress protein	Adaptation to atypical conditions
DNAK	1.7	class I heat-shock protein (chaperone)	Protein folding
pycA	1.7	pyruvate carboxylase	Metabolism of carbohydrates and related molecules; main glycolytic pathways
dltA	1.5	D-alanyl-D-alanine carrier protein ligase	Cell wall
trxA	1.5	thioredoxin	Membrane bioenergetics
splB	-2.0	spore photoproduct (thymine dimer) lyase	Sporulation
xpaC	-2.0	hydrolysis of 5-bromo 4-chloroindolyl phosphate	Metabolism of phosphate
tenI	-2.1	transcriptional activator of extracellular enzyme genes	RNA synthesis; regulation
nasC	-2.1	assimilatory nitrate reductase (catalytic subunit)	Metabolism of amino acids and related molecules
purE	-2.1	phosphoribosylaminoimidazole carboxylase I	Metabolism of nucleotides and nucleic acids

gene	Fold[1]	Function	Functional group
glpT	-2.2	glycerol-3-phosphate permease	Transport/binding proteins and lipoproteins
bioD	-2.2	dethiobiotin synthetase	Metabolism of coenzymes and prosthetic groups
spoIVFA	-2.2	inhibition of SpoIVFB and hypothesised to stabilize the thermolabile spoIVFB product (stage IV sporulation)	Sporulation
sqhC	-2.3	squalene-hopene cyclase	Metabolism of lipids
ribR	-2.3	monofunctional riboflavin kinase	RNA synthesis; regulation
pps	-2.3	phosphoenolpyruvate synthase	Metabolism of carbohydrates and related molecules; specific pathways
lipB	-2.3	extracellular lipase	Metabolism of lipids
gerM	-2.3	germination (cortex hydrolysis) and sporulation	Germination
nucA/comI	-2.3	membrane-associated nuclease	Metabolism of nucleotides and nucleic acids
spoVFB	-2.4	dipicolinate synthase subunit B	Sporulation
mmgD	-2.4	citrate synthase III	Metabolism of carbohydrates and related molecules; TCA cycle
cwlJ	-2.4	cell wall hydrolase	Cell wall
hisP/mdgS	-2.5	methionine - methionine sulfoxide transport system ATP-binding protein	Transport/binding proteins and lipoproteins
mmgA	-2.5	acetyl-CoA acetyltransferase	Metabolism of lipids
blyA	-2.6	N-acetylmuramoyl-L-alanine amidase, peptidoglycan hydrolase	Phage-related functions
ebrB	-2.6	SMR-type multidrug efflux transporter	Transport/binding proteins and lipoproteins
thyB	-2.7	thymidylate synthase B	Metabolism of nucleotides and nucleic acids
licH	-2.7	6-phospho-beta-glucosidase	Metabolism of carbohydrates and related molecules; specific pathways
hmp	-2.8	flavohemoglobin	Membrane bioenergetics (electron transport chain and ATP synthase)
csfB	-2.9	sigma-F transcribed gene	Miscellaneous
cotJC	-3.0	polypeptide composition of the spore coat	Sporulation
fabHB/yhfB	-3.2	beta-ketoacyl-acyl carrier protein synthase III	Metabolism of lipids
cotE	-3.6	spore coat protein (outer)	Sporulation
spoIIIAH	-3.6	mutants block sporulation after engulfment	Sporulation
bceB/ytsd	-3.8	bacitracine efflux ABC transport system permease	Transport/binding proteins and lipoproteins

gene	Fold[1]	Function	Functional group
Unknown genes			
yvfW	3.2	iron-sulfur-binding protein	Similar to unknown proteins (from other organisms)
yvqH	2.6	similar to phage shock protein	Similar to unknown proteins (from *B. subtilis)*
ykoJ	2.1	conserved protein	Similar to unknown proteins (from *B. subtilis)*
ydbK	2.0	probable ABC transport system permease protein	Similar to unknown proteins (from *B. subtilis)*
ywjD	-2.3	similar to UV-endonuclease	DNA restriction/modification and repair
yhfC	-2.5	conserved membrane protein, probably involved in fatty acid synthesis	No similarity to other proteins
ywdL	-2.5	bacterial regulatory helix-turn-helix protein (LysR family)	No similarity to other proteins
yomC	-2.6	N-acetylmuramoyl-L-alanine amidase, peptidoglycan hydrolase	Cell wall
yxjO	-2.6	probable transcriptional regulator (LysR family)	RNA synthesis; regulation
yckI	-2.8	probable polar amino acid transport system ATP-binding protein	Transport/binding proteins and lipoproteins
yhfU	-2.8	flavohemoglobin	Metabolism of coenzymes and prosthetic groups
ytnJ	-2.8	probable methionine sulfoxide oxidase	Detoxification
yvbA	-2.9	transcriptional regulator (ArsR family)	RNA synthesis; regulation
yhcN	-2.9	inner spore membrane protein	Similar to unknown proteins (from other organisms)
yuiF	-3.0	unknown; putative transporter	Similar to unknown proteins (from other organisms)
ycdA	-3.0	unknown	No similarity to other proteins
yjhA	-3.0	unknown	No similarity to other proteins
yngI	-4.1	long-chain fatty-acid-CoA ligase	Metabolism of lipids
ysxE	-5.2	conserved protein	No similarity to other proteins
yosA	-6.2	SP-beta protein	No similarity to other proteins

Table 4. Differentially expressed genes in response to AmyQ overproduction in the stationary phase of growth.
[1]) Fold represents relative change in expression levels between the stressed and non-stressed cells.

Gene	Fold[1]	Product	Functional group
htrA	8.8	serine protease (heat-shock protein)	Adaptation to atypical conditions
citM	6.1	2-oxoglutarate dehydrogenase (dihydrolipoamide transsuccinylase, E2 subunit)	Transport/binding proteins and lipoproteins
htrB	4.7	serine protease Do (heat-shock protein)	Adaptation to atypical conditions
fliF	4.3	flagellar basal-body M-ring protein	Motility and chemotaxis
flgB	3.8	flagellar basal-body rod protein	Motility and chemotaxis
flgC	3.6	flagellar basal-body rod protein	Motility and chemotaxis
fliJ	3.3	flagellar protein	Motility and chemotaxis
fliG	2.9	flagellar motor switch protein	Motility and chemotaxis
fliZ	2.9	flagellar protein	Motility and chemotaxis
fliE	2.9	flagellar hook-basal body protein	Motility and chemotaxis
fliY	2.8	flagellar motor switch protein	Motility and chemotaxis
PrpC/yloO	2.8	PP2C protein phosphatase	Protein modification
ilvD	2.7	dihydroxy-acid dehydratase	Metabolism of amino acids and related molecules
fliI	2.7	flagellar-specific ATP synthase	Motility and chemotaxis
fliL	2.7	flagellar protein	Motility and chemotaxis
sucC	2.5	succinyl-CoA synthetase (beta subunit)	Metabolism of carbohydrates and related molecules; TCA cycle
serA	2.5	phosphoglycerate dehydrogenase	Metabolism of amino acids and related molecules
alsT	2.5	amino acid carrier protein	Transport/binding proteins and lipoproteins
flgE	2.5	flagellar hook protein	Motility and chemotaxis
soj/spo0JA	2.4	centromere-like function involved in forespore chromosome partitioning / negative regulation of sporulation initiation	Sporulation
fliK	2.4	flagellar hook-length control	Motility and chemotaxis
fliH	2.4	flagellar assembly protein	Motility and chemotaxis
dppA	2.4	D-alanyl-aminopeptidase	Transport/binding proteins and lipoproteins
cheV	2.3	modulation of CheA activity in response to attractants	Motility and chemotaxis
TatAd/yczB	2.3	component of the twin-arginine translocation pathway	Protein secretion
flhA	2.2	flagella-associated protein	Motility and chemotaxis
uxaC/yjmA	2.2	glucuronate isomerase, hexuronate utilization	Metabolism of carbohydrates and related molecules

Gene	Fold[1]	Product	Functional group
sigD	2.1	motility, chemotaxis and autolysis sigma factor	RNA synthesis; initiation
flhP	2.1	flagellar hook-basal body protein	Motility and chemotaxis
spo0E	2.1	negative sporulation regulatory phosphatase	Sporulation
dltA	2.1	D-alanyl-D-alanine carrier protein ligase	Cell wall
rpsP	2.1	ribosomal protein S16	Protein synthesis; ribosomal proteins
mcpB	2.0	methyl-accepting chemotaxis protein	Motility and chemotaxis
smf	2.0	DNA processing Smf protein homolog	DNA packaging and segregation
nprE	2.0	extracellular neutral metalloprotease	Metabolism of amino acids and related molecules
fliD	2.0	flagellar hook-associated protein 2	Motility and chemotaxis
fhuC	2.0	ferrichrome transport system ATP-binding protein	Transport/binding proteins and lipoproteins
flgL	2.0	flagellar hook-associated protein 3	Motility and chemotaxis
motB	2.0	motility protein B	Motility and chemotaxis
htpG	2.0	class III heat-shock protein (molecular chaperone)	Adaptation to atypical conditions
mcpA	2.0	methyl-accepting chemotaxis protein	Motility and chemotaxis
qoxA	2.0	cytochrome aa3 quinol oxidase subunit II	Membrane bioenergetics (electron transport chain and ATP synthase)
qoxB	1.9	cytochrome aa3 quinol oxidase (subunit I)	Membrane bioenergetics (electron transport chain and ATP synthase)
flgK	1.9	flagellar hook-associated protein 1	Motility and chemotaxis
ffh	1.9	signal recognition particle-like (SRP) component	Protein secretion
recA	1.7	multifunctional SOS repair regulator	DNA recombination
degU	1.7	two-component response regulator involved in degradative enzyme and competence regulation	RNA synthesis; regulation
spoVC	-2.0	peptidyl-tRNA hydrolase (stage V sporulation protein C)	Sporulation
acoB	-2.0	acetoin dehydrogenase E1 component (TPP-dependent beta subunit)	Metabolism of carbohydrates and related molecules; specific pathways
opuBB	-2.1	choline ABC transporter (membrane protein)	Transport/binding proteins and lipoproteins
spoIIE	-2.3	serine phosphatase / asymmetric septum formation	Sporulation
spoIVFA	-2.4	inhibitor of SpoIVFB	Sporulation
glgA	-2.5	glycogen synthase	Metabolism of carbohydrates and related molecules; specific pathways
sspF	-2.6	small acid-soluble spore protein	Sporulation
scoB/ yxjE	-2.6	3-oxoacid CoA-transferase subunit B	Metabolism of lipids
spoVM	-2.9	required for normal spore cortex and coat synthesis	Sporulation

Gene	Fold[1]	Product	Functional group
sspD	-4.8	small acid-soluble spore protein	Sporulation
spoIIIC	-5.0	RNA polymerase sporulation-specific sigma factor(sigma-K)	RNA synthesis; initiation
spoIVCB	-5.5	RNA polymerase sporulation mother cell-specific (late) sigma factor	RNA synthesis; initiation
sspL	-5.6	small acid-soluble spore protein (minor)	Sporulation
spoVAA	-6.1	sporulation protein VAA	Sporulation
cotK	-6.2	small acid-soluble spore protein	Sporulation
spoIVB	-9.5	serine peptidase	Sporulation
tlp	-9.7	small acid-soluble spore protein (thioredoxin-like protein)	Membrane bioenergetics (electron transport chain and ATP synthase)
<u>*Unknown genes*</u>			
yloA	11.2	unknown; similar to fibronectin-binding protein	Adaptation to atypical conditions
ykoJ	5.3	conserved protein	Similar to unknown proteins (from *B. subtilis)*
yheN	3.0	similar to endo-1,4-beta-xylanase	Metabolism of carbohydrates and related molecules; specific pathways
ylxF	2.6	conserved protein	No similarity to other proteins
yfmT	2.6	similar to benzaldehyde dehydrogenase	Metabolism of carbohydrates and related molecules; specific pathways
yusS	2.5	similar to 3-oxoacyl- acyl-carrier protein reductase	Metabolism of lipids
yusQ	2.3	similar to 4-oxalocrotonate tautomerase	Metabolism of lipids
yqzH	2.2	function unknown and unique	No similarity to other proteins
yxiL	2.2	function unknown and unique	No similarity to other proteins
yopS	2.2	possible transcriptional regulator, SP-beta protein	Similar to unknown proteins (from *B. subtilis)*
ylxH	2.1	similar to flagellar biosynthesis switch protein	Motility and chemotaxis
yolA	2.1	SP-beta protein	No similarity to other proteins
yybN	2.1	function unknown and unique	No similarity to other proteins
yyzE	2.0	putative PTS glucose-specific enzyme IIA component	Transport/binding proteins and lipoproteins
ymfC	2.0	unknown; similar to transcriptional regulator (GntR family)	RNA synthesis; regulation
yorD	2.0	cold shock and salt stress induced protein	No similarity to other proteins
ycbP	-2.1	conserved membrane protein	Similar to unknown proteins (from *B. subtilis*)
yjmG/exuT	-2.0	hexuronate transporter	Transport/binding proteins and lipoproteins
yjmF	-2.1	similar to D-mannonate oxidoreductase, hexuronate utilization	Metabolism of carbohydrates and related molecules

Gene	Fold[1]	Product	Functional group
yjmE	-2.3	mannonate dehydratase, hexuronate utilization	Metabolism of carbohydrates and related molecules; specific pathways
yhfN	-2.6	putative Zn-dependent protease	Similar to unknown proteins (from other organisms)
yjmC	-2.8	similar to malate dehydrogenase, hexuronate utilization	Metabolism of carbohydrates and related molecules; TCA cycle
yxjC	-3.0	conserved membrane protein	Similar to unknown proteins (from *B. subtilis)*
yoaR	-3.2	unknown; similar to unknown proteins	No similarity to other proteins
yngG	-4.5	similar to hydroxymethylglutaryl-CoA lyase	Metabolism of lipids